



Live Webinar Q&A Sheet:

**Advances in high-throughput screening of aggregation, stability and viscosity utilizing light scattering in microwell plates**

The recorded webinar may be viewed from the [Biotherapeutics webinars page](#). These questions were submitted by live viewers. Additional information on MALS, DLS, and FFF may be found on the Wyatt web [Library](#) under webinars, application notes, featured publications and bibliography, as well as on the corresponding [Product page](#) and [Theory](#) page of our web site.

Please contact [info@wyatt.com](mailto:info@wyatt.com) with any additional questions.

Q: *Usually we calibrate MALS static light scattering with toluene, but we cannot use toluene in plastic plates. How is SLS calibrated in plates?*

A: We utilize 40 kDa dextran in a carefully prepared concentration series spanning 1-10 mg/mL. The scattering intensities are fit to the light scattering equation including second virial coefficient:  $K^*c/R = 1/M + 2A_2c$  to ensure an accurate calibration constant. The lot-certified dextran standard is provided with the instrument in a quantity that should suffice for several years of use.

Q: *What is the sample quantity and concentration required for  $A_2$  or  $k_D$  measurement?*

A: We recommend a series of 10 concentrations spanning 1 mg/mL to 10 mg/mL. The total sample quantity depends on the plate used: for a 384-well plate filled with 50  $\mu$ L per well, the total sample is 2.75 mg, while for a 1536-well plate filled with 5  $\mu$ L per well the total sample is 275  $\mu$ g.

Q: *How can the [DynaPro® Plate Reader](#) be used to characterize high-concentration antibody solutions?*

A: The primary application is determination of the solution viscosity by spiking the sample with beads of known size, such as 100 nm polystyrene latex

beads or Au nanoparticles, and measuring the apparent radius by [DLS](#) assuming viscosity of the buffer alone; the ratio between the apparent radius and true radius gives the viscosity of the protein solution. Advanced users with access to the [CALYPSO](#) software can analyze SLS measurements over a concentration series to characterize protein-protein interactions.

Q: *How do you actually discriminate between good and undesirable data point, e.g. the dust interference, etc.*

A: There are various options for automated or manual filtering of data points based on the autocorrelation amplitude and baseline value, goodness of fit (sum of squares), and reasonable maximum or minimum values. This material is covered in Dynamic Light Scattering University ([DynaLSU](#)), Wyatt's training course for theoretical and hands-on applications of dynamic and electrophoretic light scattering.

Q: *Any insights on correlation between  $k_D$  and opalescence?*

*Is the instrument able to accurately determine  $k_D$  on opalescent sample? The data quality and correlograms for these samples are difficult to interpret and don't trend with concentration consistently.*

A: Opalescence may arise from distinct physical effects including phase separation (forming protein-rich microscopic droplets dispersed in low-protein solution) and fibrillization. The diffusion interaction parameter  $k_D$  can be measured on pre-opalescent samples, i.e. at concentrations below the onset of opalescence. Then it is a good predictor of opalescence at higher concentrations and can be used to screen for buffers that are less prone to opalescence.

However, it is not a good idea to attempt to measure  $k_D$  under conditions that already exhibit opalescence, where the optical signal is some combination of light scattered from interacting monomeric proteins and the larger proteinaceous structures such as droplets or fibrils, and can be expected to be difficult to interpret.

Q: *Could we use the DynaPro Plate Reader III to optimize buffer screens for protein complexes?*

A: Yes, absolutely! A clean experiment will detect changes in average size of 1-2% and changes in average molar mass of ~ 10%. A protein complex such as a homodimer will differ in size from the monomer by about 40% and in molar mass by 100%, and larger complexes such as trimers will differ by even more. As the fraction of complex in the solution increases due to buffer conditions that favor complex formation, this will readily be detected as a shift in average size or molar mass. Just such measurements are presented in Hanlon et al. (2010) "Free-Solution, Label-Free Protein-Protein Interactions Characterized by Dynamic Light Scattering". *Biophys. J.* **98**(2), 297-304

Q: *Is the size distribution quantitative? Can I know quantitatively how much protein/antibody is aggregated and how much is monomer? If yes, can it replace SEC for aggregation quantitation or screening highly-aggregated in-process antibody products?*

A: SLS does not provide size distributions, just a single weight-average molar mass value for the solution. DLS does provide size distributions, but not high-resolution quantitative size distributions comparable to SEC or [SEC-MALS](#), and will not provide accurate values of % aggregate vs. %monomer, especially if you are focusing on small aggregates such as dimer-trimer. However, DLS can provide these properties for estimating aggregate populations:

1. **Polydispersity:** the width of the mode containing the monomer and small oligomers, which increases as more oligomers form (but in principle could be low if all the monomers have dimerized)
2. **Average radius:** the average value of the mode containing the monomer and small oligomers, which increases as more oligomers form (it is best to combine average with polydispersity to analyze oligomeric content)
3. **Large aggregates populations:** aggregates larger in radius than the monomer-dimer population radius by at least ~ 3-5x will present a distinct mode (peak) in the DLS size distribution. However, aggregates of this size will typically be filtered out or disrupted by the SEC column and will not appear in the chromatogram, so DLS is a good way to identify and quantify them. Typically the fraction of large aggregate is quantified as %Mass or %Number.

Q: *What is the concentration range that can be used for the plate?*

A: The concentration range depends on the molar mass of the protein and the type of measurement desired (DLS or SLS). For IgG, the lower concentration limit of DLS is 12.5 µg/mL and of SLS is 400 µg/mL. For lysozyme, which has a molar mass 1/10 that of IgG, the lower concentration limit of DLS is 125 µg/mL and of SLS is 4 mg/mL. There is no restriction on the upper limit for measurement but data interpretation can become quite tricky above ~15-20 mg/mL for any protein.

Q: *Why is the hydrodynamic radius of an IgG in histidine buffer only half size compared to the other buffer in your slide?*

A: DLS measures the translational diffusion coefficient  $D_t$  and then converts that to an apparent hydrodynamic radius  $r_h$  using the Stokes-Einstein equation (see [DLS Theory](#)). As concentrations increase, protein-protein interactions modulate  $D_t$ : attractive interactions reduce  $D_t$  and appear as a larger  $r_h$ , while repulsive interactions increase  $D_t$  and appear as a lower  $r_h$ . The low apparent value of  $r_h$  for IgG in low-ionic-strength histidine buffer is a result of strong electrostatic repulsion since the buffer does not shield the charges on the molecules. This effect is captured in the diffusion interaction parameter measurement which plots the apparent  $D_t$  vs. protein concentration.

Q: *How can you determine a  $k_D$  at temperature of e.g. 70°C when aggregates are scattering?*

A: Values of  $k_D$  under conditions where aggregates have begun to form should be ignored, since they include effects of aggregation kinetics as well as thermodynamics and hydrodynamics. Only those values at temperatures below the onset of aggregation are reliable and useful.

Q: *If  $T_m$  is not the best way to predict aggregation, why do many scientist use it as a gold standard?*

A: Proteins are complicated, degradation pathways diverse, and predicting aggregation is not yet hard science. In the literature,  $T_m$  sometimes correlates well with accelerated stress at elevated temperature or freeze-thaw, and sometimes not. It has not been found to correlate well with long-term stability of

IgG under typical storage conditions – see e.g. Razinkov et al. (2015) “Accelerated Formulation Development of Monoclonal Antibodies (mAbs) and mAb-Based Modalities: Review of Methods and Tools”, *J. Biomol. Screening* **20**(4):468-83 and Brader et al. (2015), “An examination of thermal unfolding and aggregation profiles of a series of developable therapeutic monoclonal antibodies”, *Mol. Pharm.* **12**(4):1005-17. Perhaps the best reason that scientists continue to hold this property in high regard is tradition... Sophisticated researchers typically combine  $T_m$  analysis with other attributes such as  $A_2/k_D$ , rate of aggregation at elevated temperature, quantity of aggregates formed under freeze-thaw and other stress conditions, and computational approaches to predict propensity for aggregation.

Q: *What are orthogonal techniques that can be used to validate the results of a formulation screen with DLS and SLS?*

A: UHP-SEC-MALS (UHPLC-SEC plus a  [\$\mu\$ DAWN](#)) is ideal for validating results that exhibit small oligomerization via increases in polydispersity and/or shift of the average values of  $r_h$  and  $M_w$ . For larger aggregates, FFF-MALS is appropriate as it can separate and characterize aggregates up to 1000 nm. If the formulation screen indicates self-association or the  $A_2$  measurements need validation, [CG-MALS](#) using a [Calypso® II](#) composition-gradient system and Wyatt MALS detector such as [DAWN](#) or [miniDAWN](#) provides highly accurate measurements and analyses of these properties.

Q: *What are the best strategies to characterize 20 nm aggregates?*

A: DLS/SLS is a good start, especially in combination with  $A_2/k_D$  analysis to characterize equilibria and protein-protein interactions. If the aggregates are stable under dilution and shear then the next step would be SEC-MALS-DLS. If the aggregates are stable under dilution but not shear then [FFF-MALS](#) is appropriate. A last resort would be analytical ultracentrifugation/sedimentation velocity, which does not dilute or shear the aggregate but is slow, requires manual operations and an AUC expert to interpret the results.